Estrogen Derivatives of Transition-Metal Complexes for Analytical Detection Enhancement†

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We report herein the labeling of 17α-ethynyl-17β-estradiol with a "$Co_3(CO)_9$" fragment. The cluster exhibits fully reversible electrochemical behavior and is assayed by means of the square-wave voltammetric technique. During the condensation of metallocarbonyl fragments around the organic chain, elimination of the 17β-OH group in the original molecule occurs, affording $Co_3(CO)_9$(dehydroxyethynylestradiol). This modification causes an important decrease of relative binding affinity for the receptors. However, the cluster can still be used for immunoassay of estradiol, since its cross reactivity for specific antibodies is high.

Introduction

The labeling of steroids can be used either in immunology for assaying circulating hormones involved in some specific pathologies or in receptorology for quantifying the levels of specific receptor of these steroids (*e.g.* in hormone-dependent tumors).[1] Due to its specificity and sensitivity, immunoassay is the method of choice for trace analyses of biologically, clinically, and pharmaceutically important molecules. Radioisotopic labels (mainly ^3H, ^{14}C, and ^{125}I) can be incorporated into several biomolecules, and their relatively easy detection guaranteed the success of the radioimmunoassay (RIA) method.[2] However, the problems connected with the use of radioactive materials have stimulated a continuing effort to develop new nonisotopic (*cold*) immunoanalytical procedures.[3] The complexation of a biomolecule by a metallic tracer could represent a potential solution to this problem, providing that some degree of recognition between the new complex and the specific targets (receptors, antibodies, enzymes) was maintained. It has been observed that complexation with metallic fragments can deeply alter their biochemical reactivity. Several groups have suggested the possibility of using organometallic tracers in immunology. The detection techniques associated with these tracers were atomic absorption spectroscopy[4] and electrochemistry.[5] More recently, Jaouen used transition-metal carbonyl fragments as labeling agents for the quantitative determination of hormones.[6] In this method the protocol consists of a spectroscopic analysis of the hormone-receptor precipitate obtained after incubation of the metal-labeled steroid with cytosol. The detection is usually carried out by using FT-IR spectroscopy, since all carbonyl complexes exhibit strong ν_{CO} bands in the range 2100–1800 cm^{-1}, a region where absorption due to proteins is minimal. Accordingly, Jaouen coined the novel acronym CMIA (carbonylmetalloimmunoassay).[6]

An alternative detection mode for such a metal-labeled steroidal hormone in solution is afforded by electrochemistry. The coordination of polymetallic frames to steroids makes them easily electroreducible, since an accessible LUMO (generally metal–metal antibonding in character) is available. This additional, well-behaved reduction process is suitable for cathodic detection.[7] Unfortunately, the binuclear complex first employed, namely [$Co_2(CO)_6$(17α-ethynyl-17β-estradiol)] (**1**; Figure 1), exhibits a diffusion-controlled but chemically irreversible reduction process.[7] However, this electrochemical behavior allows the use of techniques such as polarography and linear sweep voltammetry (LSV), which provide detection limits on the order of 10^{-5} M. The rapid and very sensitive square-wave voltammetry (SWV) technique can give a better response when the compound under investigation undergoes fully reversible redox processes.[8] With the purpose of increasing the electrochemical detection limit for steroid–organometallic complexes, we report herein attempts to label 17α-ethynyl-17β-estradiol with a "$Co_3(CO)_9$" fragment, which is known to exhibit chemically reversible electrochemical behavior.[9] The assay of this labeled molecule by means of the SWV technique is described.

Results and Discussion

Synthesis and Characterization of 3. Several comprehensive reviews[10] have dealt with the synthetic

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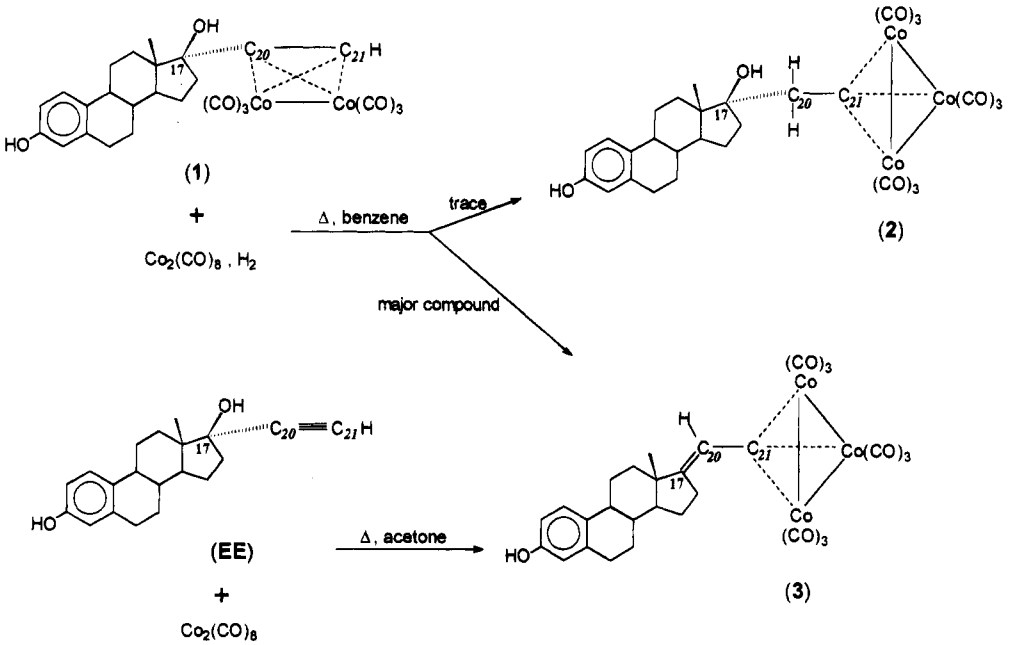

Figure 1. Sketch of the synthesis of $Co_3(CO)_9(C{-}CH_2{-}E)$ (**2**) and $Co_3(CO)_9(C{-}CH{=}E')$ (**3**) along with the numbering scheme of the key carbon atoms.

routes to (alkylydyne)tricobalt nonacarbonyl compounds, $Co_3(CO)_9CR$. The classical route employing a α,α,α-trihalo derivative does not appear feasible for estradiol. In fact, some reports[11] on the reaction of chloro or bromo alcohols, $CX_3CRR'OH$, with $Co_2(CO)_8$ show that, instead of the expected $Co_3(CO)_9(CCRR'OH)$ derivative, the dehydroxylated product $Co_3(CO)_9(CCHRR')$ is obtained, albeit in low yield. The simplest way to obtain $Co_3(CO)_9(C{-}CH_2{-}E)$ (**2**; E = estradiol radical = $C_{18}H_{23}O_2$) should be the treatment of the dicobalt complex **1** with H_2 and $Co_2(CO)_8$ under mild conditions (benzene, 65 °C).[12] Indeed, this reaction produces some $Co_3(CO)_9CR$ derivatives (overall yield ca. 20%) (Figure 1). The presence of the "$Co_3(CO)_9C$" moiety is clearly shown by the typical five-band IR spectrum in the carbonyl stretching region[12] and by the reversible cyclic voltammetric response at $E^{\circ\prime} = -0.700$ V vs SCE.[9] However, NMR spectroscopy clearly indicates a mixture of quite similar $Co_3(CO)_9CR$ derivatives (inseparable by the usual chromatography and crystallization workup procedures). The less abundant compound (ca. 10% by ^1H NMR integration) likely corresponds to the originally desired $Co_3(CO)_9(C{-}CH_2{-}E)$ (**2**) cluster, as identified by the multiplet (AB system) centered at 3.72 ppm (*i.e.* the methylenic group in $Co_3(CO)_9C{-}Et$ exhibits a quartet centered at 3.77 ppm[13]). The most abundant compound unambiguously (*vide infra*) corresponds to the $Co_3(CO)_9(C{-}CH{=}E')$ derivative (**3**; E'=17β-dehydroxyestradiol radical, $C_{18}H_{22}O$) (Figure 1). During the formation of **3** elimination of the 17β-OH group has occurred, and a 1,2-shift of hydrogen results in the generation of a $C_{17}{-}C_{20}$ double bond. Due to the impossibility of separating **2** and **3** by chromatography, and to considerably improve the yield of **2** in the product mixture by increasing the H_2 pressure (at least up to 4 bar of pressure[12]), we optimized the synthesis of **3** alone, simply by reacting $Co_2(CO)_8$ with EE in acetone at reflux for 20 h (ca. 30% yield) (Figure 1). Once **3** was obtained in pure form, the characterization was straightforward. The mass spectrum of **3** in the desorption chemical ionization (DCI) mode, with CH_4 as reagent gas, showed the ion $[M + H]^+$ at *m/z* 709, and a very abundant fragment ion corresponding to the dehydroxyethynylestradiol cation, $[C_{20}H_{23}0]^+$, when positive ions were collected. In contrast, when negative ions were collected, the highest ion at *m/z* 680 corresponded to [M − CO], the usual behavior for metal carbonyl clusters.[14] In the ^1H NMR spectrum the singlet due to 17-OH (at ca. 4.3 ppm in free EE) was absent while a singlet appeared in the olefinic zone (7.65 ppm). The remaining signals did not change considerably when compared to those of free EE. The $^{13}C\{^1H\}$ NMR spectrum of **3** showed a resonance at 275.3 ppm unambiguously assigned to the apical carbon atom bonded to three cobalt atoms,[15] namely C_{21}, while the intense resonance at 200.3 ppm is typical of the carbonyls of the $Co_3(CO)_9$ fragment undergoing rapid scrambling[15] (Figure 2). A new resonance appeared in the olefinic region (at 139.6 ppm): in the proton-coupled ^{13}C NMR spectrum this signal became a doublet ($^1J_{C-H}$ = 152 Hz) and was selectively decoupled by irradiation at the frequency of the hydrogen peak at 7.65 ppm, thus confirming the assignment to an olefinic C—H group. Finally, the C_{17} resonance was shifted from 87.6 ppm in free EE[16] to 152.7 ppm in **3**. This large downfield shift confirmed the strong interaction between this carbon atom and the $Co_3(CO)_9$ unit. Several attempts to grow crystals suit-

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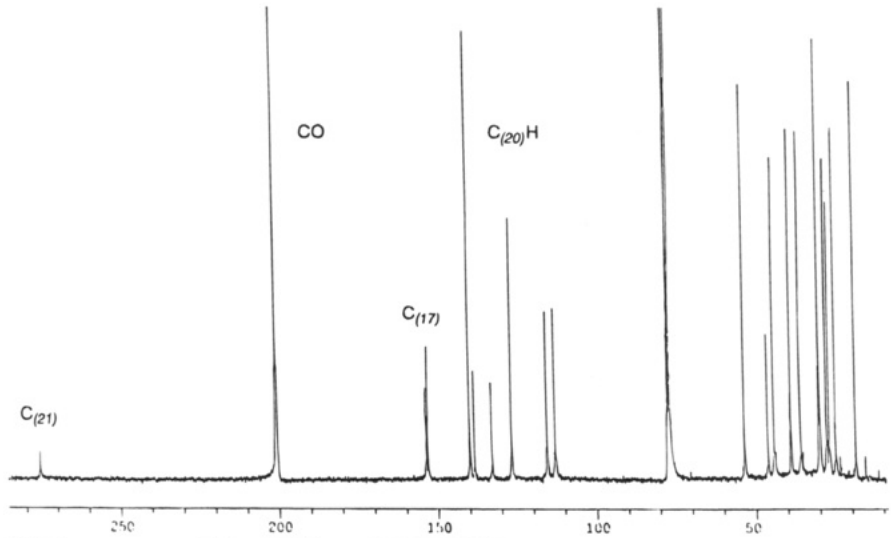

Figure 2. $^{13}C\{^1H\}$ NMR spectrum of **3** in $CDCl_3$ at 100.577 MHz.

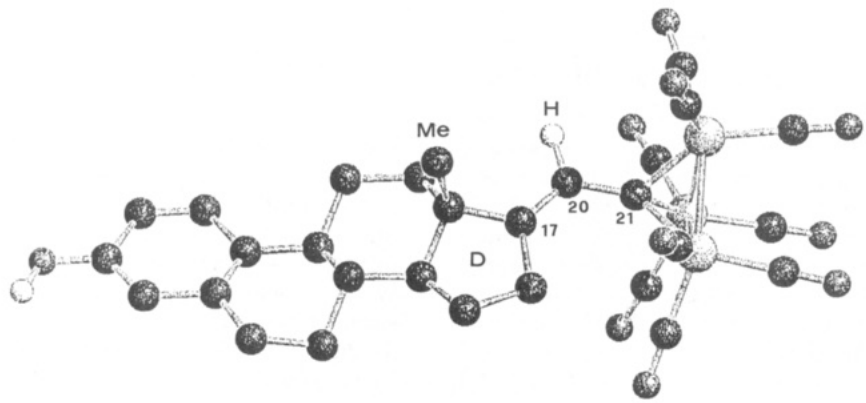

Figure 3. SCHAKAL plot of the structure of **3** (trans isomer) obtained by means of the molecular modeling program PCMODEL,[17] along with the numbering scheme of the key carbon atoms.

able for X-ray determination have been unsuccessful to date. A reasonable representation of the structure of **3** can be obtained by the use of the molecular modeling program PCMODEL.[17] The organometallic fragment $Co_3(CO)_9C_{ap}$—C_{ipso} is derived from the X-ray structure of $Co_3(CO)_9(C_{ap}$—Ph)[18] and the E′ fragment from the estradiol−propanol adduct,[19] where the 17β-OH functionality is removed and the 17α-H atom replaced with the $-C_{20}(H)-$ group at 1.34 Å. The two fragments are then combined as in **3** (C_{ap} coincides with C_{21} and C_{ipso} with C_{20}). Due to the C_{17}=C_{20} double bond and the asymmetry of ring D in the steroid, two geometrical isomers can be generated. The initial structures of both isomers are energy-minimized by allowing small oscillations about the C_{20}—C_{21} bond to find the most favorable conformation. The *trans* isomer reaches a lower energy minimum as expected, since the synthesis of **3** is stereospecific, as confirmed by the NMR spectra. The resulting *trans* structure is depicted in Figure 3, making use of the SCHAKAL program.[20] While recognizing the crude approximations inherent in such a procedure (*e.g.*, the fixed structure of the metal carbonyl fragment), we feel that this provides a reasonable model for the most stable stereoisomer.

Biochemical Results. We have determined the relative binding affinity (RBA) value of **3** for the estradiol receptor and its cross-reactivity (CR) for the anti-estradiol antibody.

(a) Binding Affinity of 3 for the Estrogen Receptor. The binding affinity of **3** for the estrogen receptor of lamb uterine cytosol has been measured by a competitive binding method, which is a convenient method for determining the binding affinity of modified hormones. The relative binding affinity (RBA) value found for **3** is only 0.4 (the RBA value of estradiol itself is taken to be 100%). This rather low RBA value is not surprising, since it has been reported that the modification of one of the 3- or 17β-OH groups of estradiol causes an important decrease of affinity for the receptors.[21] Cluster **3** can be better used for immunoassays with estradiol *antiserums*, since such polyclonal antibodies are still able to recognize the unmodified part of the coordinated biomolecule.

(b) Studies of Recognition between Antibodies and 3. We determined the cross-reactivity of **3** for the anti-estradiol antibody. The CR value was calculated

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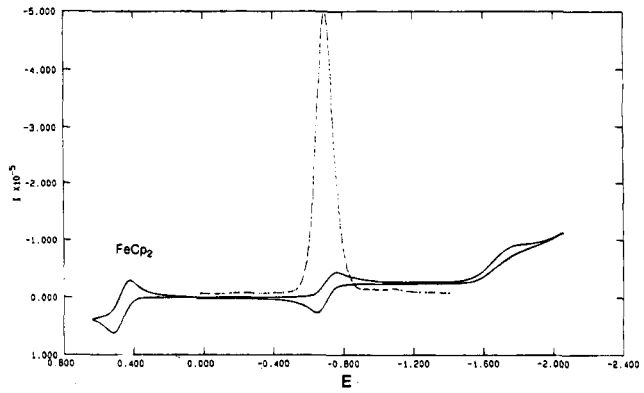

Figure 4. Cyclic voltammetric (solid line) and square-wave voltammetric (dotted line) responses of a CH_2Cl_2 solution (containing $[NBu_4][PF_6]$ 0.1 M as supporting electrolyte) of **3** at an HMDE. $E^{\circ\prime} = E_{1/2} = (E_p^a + E_p^c)/2 = E_{su}$(SWV) = −0.700 V vs SCE. The ratio between i_{su}(SWV) and i_p(CV) is ca. 15.

from competition curves which give the quantities of **3** that displace by 50% the binding of the radioactive estradiol. The CR (%) is equal to the ratio (×100) of the test compound which displaces by 50% the binding of the corresponding radioligand over the quantity of estradiol also giving 50% displacement. By definition, the CR for the reference compound (*i.e.* estradiol) is equal to 100%. The CR value found for **3** was 62%. This quite high value indicates that **3**, in spite of the lack of a 17β-OH group, can be used as a reagent for such an immunoassay.

Electrochemical Assay of 3. The electrochemical behavior of **3** in CH_2Cl_2 has been studied by means of dc polarography, cyclic voltammetry (CV), and square-wave voltammetry (SWV). The electrochemical scenario is similar to that previously reported for other $Co_3(CO)_9$-CR analogues[9] and consists of a fully reversible 1e reduction at $E^{\circ\prime}(0/1-) = -0.700$ V vs SCE in CH_2Cl_2, followed by a further reduction, chemically irreversible, at $E_p(1-/2-) = -1.73$ V vs SCE (estimated from the CV response at the scan rate 0.20 V s^{-1}) (Figure 4). The electrochemical reversibility of the first reduction (fast electron transfer process) was established by the 63-mV slope of the E vs $\log[(i_d - i)/i]$ plot obtained from polarography, by the 65-mV value of $\Delta E_p = E_p^a - E_p^c$, obtained from CV, and by the 95-mV value of peak width at half-height, $W_{1/2}$, obtained from SWV. The chemical reversibility of the process (persistence of the electrogenerated monoanion $\mathbf{3}^-$) was assessed by the value of i_p^a/i_p^c obtained from CV, which was constantly equal to unity in the scan rate range 0.05−50 V s^{-1}, and by the complementary polarographic responses before and after 1e exhaustive electrolysis. Noteworthy is the coincidence within experimental error (±5 mV) of the polarographic $E_{1/2}$ potential, evaluated from the E vs $\log[(i_d - i)/i]$ plot, the CV $E^{\circ\prime}$ potential, evaluated as $(E_p^a + E_p^c)/2$, and the summit potential E_{su} from SWV. This points to a full reversibility of the 0/1− process. The use of Pt in place of Hg as an electrode material does not change significantly the electrochemical scenario, and neither does the use of acetone in lieu of dichoromethane as solvent. In this case, however, the reduction potentials shift toward less cathodic values, as expected for a more polar, better solvating solvent: $E^{\circ\prime}(0/1-) = -0.54$, and $E_p(1-/2-) = -1.57$ V vs SCE at 0.20 V s^{-1}.

Figure 4 shows the advantage of the SWV over the CV technique for the fully reversible 0/1− reduction of cluster **3**. The bold line represents the CV response at a hanging-mercury-drop electrode (HMDE) of a CH_2Cl_2 solution of **3** at 0.20 V s^{-1} in the available cathodic window. The dotted line represents the SWV response of the same solution at the identical electrode using 1 mV as the potential step increment (ΔE), 50 mV as the square wave amplitude (E_{sw}), and 200 Hz as the frequency (f).[8] The SWV window is centered around the first reduction potential, being the only such process that is chemically reversible. Noteworthy is the fact that the time scales of the two determinations are identical, since in SWV the scan sweep can be evaluated by $f\Delta E$, this value being 0.20 V s^{-1}. The ratio i_{su}(SWV)/i_p(CV) is about 15. Interestingly, a further increase of sensitivity in SWV can be achieved by increasing f up to 400 Hz and E_{sw} up to 100 mV. The detection limit of **3** in such SWV experimental conditions reaches 10^{-7} M; since the volume of the electrochemical microcell is 1 mL, this implies a minimum detectable quantity (MDQ) of nanomole order. This sensitivity is enough for any pharmacological analyses.[22]

Experimental Section

IR and NMR spectra were recorded on a Perkin-Elmer 580 B and on a JEOL-EX 400 spectrometer, respectively. Elemental analyses were carried out in Torino.

Desorption chemical ionization (DCI) MS spectra were recorded on a Finnigan MAT 95Q instrument with both magnetic and electrostatic analyzers. Methane was used as the reagent gas at 0.5 Mbar pressure. The ion source temperature was kept at 50 °C, the electron emission current at 0.2 mA, and the electron energy at 200 eV. Both positive and negative ion spectra were recorded.[23]

Electrochemical measurements were performed using an EG&G PAR 273 electrochemical analyzer interfaced to an IBM computer, employing PAR M270 electrochemical software. A standard three-electrode cell was designed to allow the tip of the reference electrode (SCE) to closely approach the working electrode. Positive feedback iR compensation was applied routinely. All measurements were carried out under prepurified Ar in anhydrous deoxygenated solvents. The working electrode for CV and SVW was a hanging-mercury-drop electrode (HMDE, Metrohm Model No. 6.0335) or a Pt-disk electrode (area ca. 0.8 mm^2) embedded in a Teflon seal.

Preparation of $Co_3(CO)_9(C-CH=E')$ (3). The synthesis of **3** was brought about by heating an equimolar mixture of 17α-ethynyl-17β-estradiol (EE) and $Co_2(CO)_8$ in refluxing acetone for 20 h. The reaction was followed by TLC on Kieselgel (eluent 20/80 ether/hexane). $Co_2(CO)_6(EC{\equiv}CH)$ (**1**) was first formed and then smoothly turned into **3**, which gave a fast-moving violet spot. Chromatography on a silica gel column with the same eluent and evaporation of the solvent mixture under reduced pressure yielded violet-black microcrystals (ca. 30%). IR (hexane): ν_{CO} 2100 (m), 2052 (vs), 2038 (s), 2018 (w), 1988 (vw) cm^{-1}. ^1H NMR (acetone-d_6): δ 8.14 (s, C_3—OH), 7.65 (s, C_{20}—H), 7.22 (d, C_1—H), 6.71 (dd, C_2—H), 6.66 (d, C_4—H), 3.2−1.1 (all remaining, partially overlapped, resonances), 1.03 (s, C_{17}—*Me*). ^{13}C NMR ($CDCl_3$): δ 275.3 (C_{21}), 200.3 (CO's), 153.4 (C_3), 152.7 (C_{17}), 139.6 (C_{20}, $^1J_{CH}$ = 152 Hz), 138.2 (C_5), 132.6 (C_{10}), 126.4 (C_1), 115.3 (C_4), 112.7 (C_2), 53−22 (all remaining resonances), 18.4 (C_{17}—*Me*). Anal. Calcd

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for $C_{29}H_{23}O_{10}Co_3$: C, 49.18; H, 3.27; Co, 24.96. Found: C, 49.06; H, 3.29; Co, 24.88.

Competitive Binding Assay. Aliquots (200 μL) of lamb uterine cytosol as previously described[24] were incubated for 3 h at 0 °C with 2×10^{-9} M of [^{3}H]estradiol in the presence or absence of competing unlabeled steroids (nine concentrations ranging from 1×10^{-10} to 1×10^{-6}). The percentage reduction in binding of [^{3}H]estradiol (Y) was calculated by use of the logit transformation of Y. The relative binding affinity (RBA) of the competitor is taken as the ratio of the concentrations of unlabeled estradiol/competitor required to inhibit half of the specific [^{3}H]estradiol binding with the affinity of the estradiol set at 100%.

Competition Curves by Radioimmunoassay. The antibody used was frozen polyclonal anti-estradiol antiserums from sheep and was a gift from Société Medgenix (Fleurus, Belgium). The injected antigen was 3-(carboxymethyl)estradiol oxime synthesized by the method of Erlanger.[25] Fractions (1 mL) containing 50 μL of antibody (final dilution 1/1000), 50 μL of [^{3}H]estradiol (0.17 pmol), and increasing amounts of either estradiol or **3** in phosphate buffer were incubated for 1 h at room temperature. At the end of the incubation the free and bound fractions were separated by Dextran-coated charcoal (final concentration 1.25% of charcoal, 0.125% of dextran T70). The supernatant liquids containing the bound fractions were transferred into vials, and the radioactivity was counted on an LKB-1211 Rackbeta counter using ACS (Amersham) scintillation liquid.

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